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SOME PECULIARITIES OF THE MALE FROG TEST FOR EARLY PREGNANCY

D. F. MILLER AND P. B. WILTBERGER¹

An article describing the use of the male of the leopard frog (*Rana pipiens*) as a new test animal for early pregnancy was published in *Science* for February 20, 1948 (1). This is a rather radical departure from the widely used effects of the gonadotropic hormones upon the female sex organs of test animals. In this new test we have described a method that has as its chief virtues accuracy, rapidity, sensitivity, and simplicity.

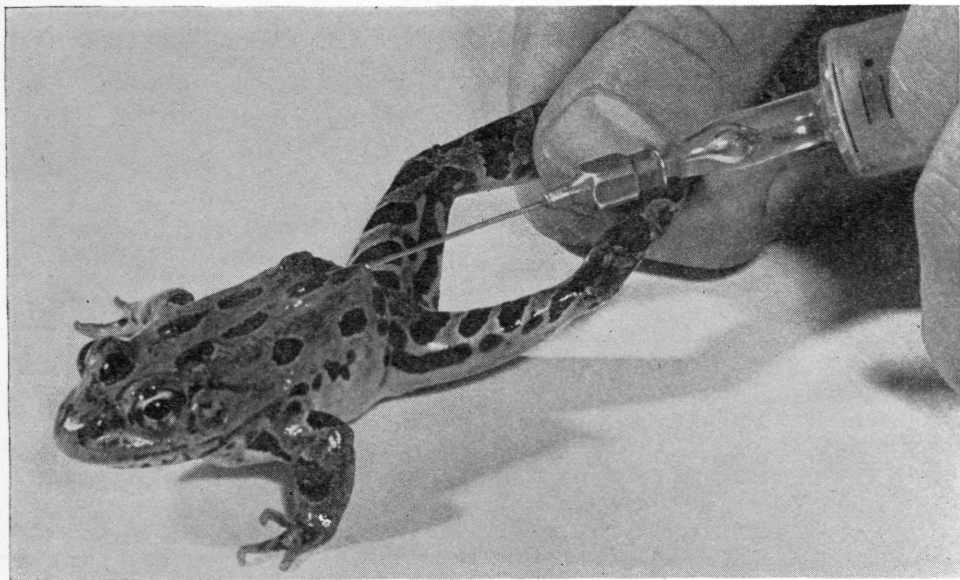


FIG. 1. Method of injecting into the dorsal lymph sac of a frog.

The simplicity of the technic is easily seen from the following description. The patient secures an ounce or more of the first urine she voids upon arising in the morning and sends this to the physician for the test. The technician injects, by hypodermic syringe, from 2 cc. to 4 cc. of the urine into the lymph sacs of a male frog using a 25 gauge needle. Larger needles may allow the urine to leak out. The frog is then placed in a pint fruit jar which has a wide mouth and a perforated lid. The jar is labelled and set aside for a period of from two to four hours during which the frog absorbs the urine. At the end of this period an examination of the

¹D. F. Miller, Chairman, Department of Zoology and Entomology, The Ohio State University, Columbus, Ohio. P. B. Wiltberger, M.D., 350 East State Street, Columbus, Ohio.

jar will often show the presence of urine voided by the frog. If not, the frog may be made to urinate by seizing its head and shoulders in the hand whereupon the frog will reflexively eject a jet of urine. A drop of the voided urine is placed upon a microscope slide covered with a cover-glass and examined under 16 mm. and 4 mm. objectives. Since the spermatozoa of *Rana pipiens* are large the technician soon learns to recognize them with the 16 mm. lense alone. The presence of the frogs sex cells in the urine indicates a positive result of the test for the presence of the gonadotropic hormone usually found in the blood plasma of pregnant women.

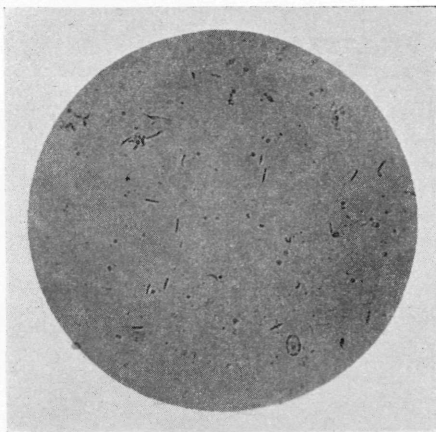


FIGURE 2

FIG. 2. Photomicrograph of a drop of frog urine containing frog sperm cells, as seen when using 10× ocular and 16 mm. objective.

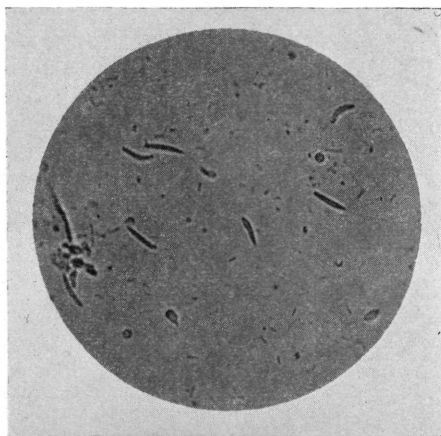


FIGURE 3

FIG. 3. Photomicrograph showing frog sperm cells as seen when using 10× ocular and 4 mm. objective. Cigar-shaped bodies are sperm heads each of which bears a transparent flagellum at one end. There are eight sperms appearing in this illustration.

The absence of the frog sperm cells is a negative result of the test for the hormone. Since the activating agent *seems* to be chorionic in origin its presence in the urine of the women should ordinarily indicate pregnancy. R. G. Hoskins (2) says that the placenta "apparently also functions as an endocrine gland producing important regulatory hormones. Concentration of the urine insures greater certainty of the results and the use of fewer frogs.

CARE AND HANDLING OF THE FROGS

The leopard (or grass) frog is widely distributed and may be bought from frog farms, biological supply houses, or collected locally. The ideal time to collect them seems to be in early autumn before cold weather has brought on hibernation. The testes of such frogs contain mature sperms. They may be kept best in a cold storage room in which the temperature never gets below freezing, from 34° to 40°F. They should be placed in a box of damp peat moss and sprinkled lightly whenever they seem to be getting dry. If a few frogs are brought out of this storage one or two days before they are used in tests they can be kept in a wet place until used. Frogs should be wet at all times but *not kept in water from which they cannot crawl out*. In deep water a frog will drown. A very satisfactory container is a pan or tray 4 inches deep by 18 inches wide by 30 inches long. It should be covered with a lid of half-inch mesh woven wire so hinged that each end of the lid may be opened without opening the whole top of the pan. Such a pan will hold up to 100 frogs

to start. If the pan is tilted so that water will stand in one end while the other is free from water the frogs can keep wet by getting into the water. If the water is not running it should be changed every day. Frogs that are cool will keep for weeks without feeding. Small crayfish, earthworms or mealworms can be fed if the frogs are not in storage, but this is hardly necessary.

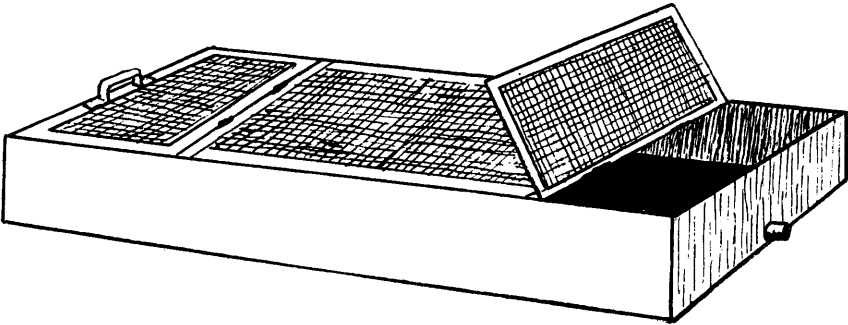


FIG. 4. Tray for keeping a stock of 100 or less frogs.

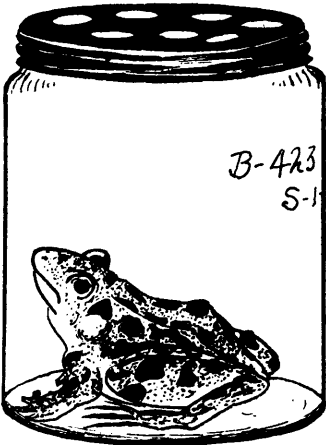


FIGURE 5

FIG. 5. Wide-mouthed pint fruit jar with perforated metal lid for holding individual frogs during test.

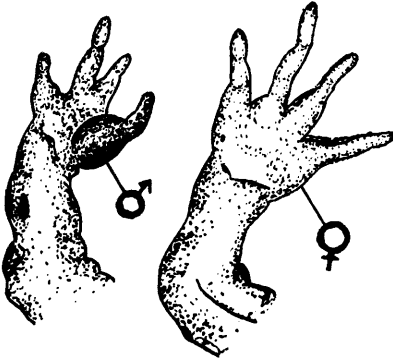


FIGURE 6

FIG. 6. Male frogs are distinguished from females by swollen base of "thumb."

If a frog has been used in a test and the test was negative, that frog may be returned to the original pan and used over again at any time. But if the test was positive the frog should be placed in another container for at least four days before being reused. Hence two pans should be provided as containers. For the individual physician who wishes merely to run an occasional test for a patient it is possible to have half a dozen frogs which can be kept in a large jar with a heavy screen top. A very thin layer of water in the bottom of the jar is sufficient and this should be changed daily.

In this species of frog (*Rana pipiens*) the sexes are easily distinguished by the difference in the appearance of the base of the first digit (thumb) on the foreleg. It is generally swollen and more heavily pigmented in the male as shown in figure 6. The body of a mature frog of this species should be from two and a half to three

and a half inches in length. Orders to supply houses or collectors should specify "males only", and even then the technician should *carefully check each frog* when it is selected for a test.

The skin of the frog is very loosely attached to the body wall, being fastened by strips of connective tissues which divide the space between the skin and the muscles into large pockets, or lymph sacs. In making an injection the needle should be thrust through the skin but not into or through the body wall. Inter-peritoneal injection can be made but is likely to result in severe injury to the frog. During the injection it is best to hold the frog by its hind feet. The frog will

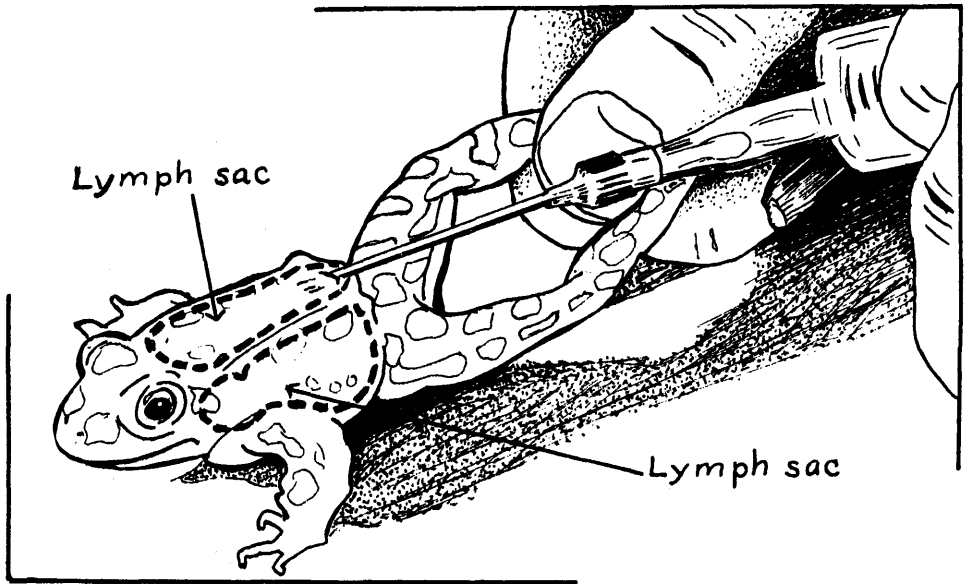


FIG. 7. Showing dorsal and lateral lymph sacs.

straighten out on the table top and does not struggle much during injection. A check on each frog can be made by placing it in a jar for an hour before it is injected. At the end of this time its urine can be examined for sperm cells. It must be negative. Then the frog may be injected for the test, each frog furnishing its own check. We have always recommended that two frogs be run for each test as a precaution against individual differences or in the case where the potency of the hormone is low.

SOME PECULIARITIES AND RESULTS OF THE TESTS

Samples of pregnancy urine may be kept and used for demonstration purposes or for producing live spermatazoa for classroom study. One sample of such urine was kept in the refrigerator for five weeks and then gave a good positive test. The same sample after standing at room temperature for six more days still gave as good a test when fresh. Another sample was kept for several days at room temperature and then preserved by adding two drops of toluene and shaking thoroughly. After standing at room temperature for eight weeks it produced a strong positive test. This seems to indicate that the *gametokinetic substance* does not deteriorate readily. However, some of the commercially prepared anterior pituitary-like substances degenerate rapidly when opened to the air. The commercially prepared substance will give a heavy discharge of sperm cells if they are

used in fresh condition. A sample of urine known to give a strong positive test can be kept and used upon a male frog at any time that living sperm cells are needed for teaching purposes. These large sperms make excellent demonstration material and may be obtained so much more readily than from mammals. Being fresh they are better teaching materials than a stained smear which is so often used. Urine for this purpose cannot be preserved by heating as heating to the boiling point destroys the effectiveness of the urine.

In a few cases we have found that a urine sample is very toxic to the frogs causing death too early to get a test. In these it has been possible to get a successful test by gradual dilutions of the urine and by watching the test animals so as to collect a urine specimen from them before death. As small a sample as 0.5 cc. of pregnancy urine diluted to 2.0 cc. with tap water has been successful in getting a positive test in some such instances. Adjusting the pH to neutrality is also helpful.

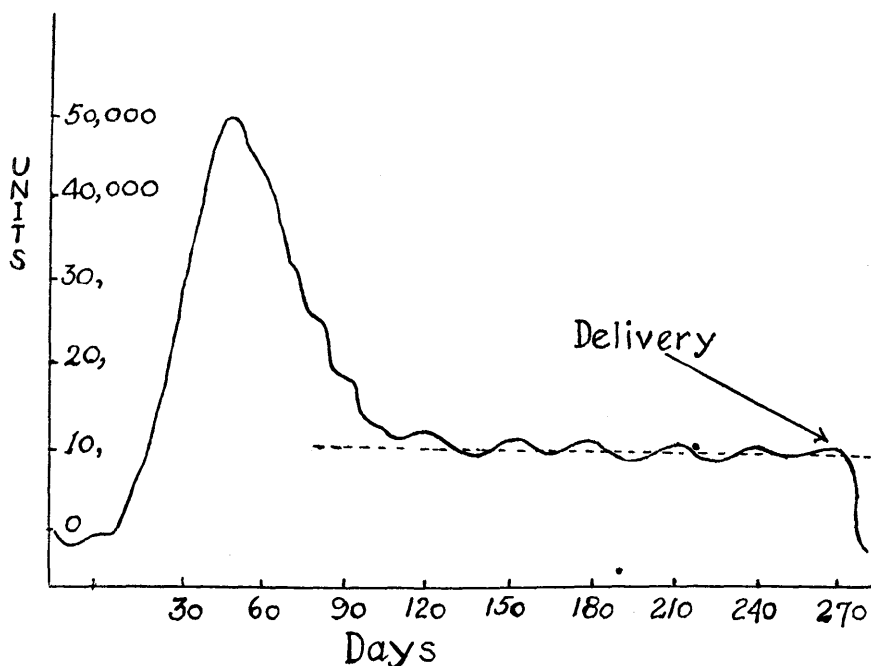


FIG. 8. A composite graph of the abundance of gametokinetic substance in urines during pregnancy. Not taken from any particular study.

In one case of uncertain pregnancy we obtained a urine sample and blood sample at the same time. The urine sample gave a positive test when run with a frog. The blood serum was used upon another frog in the same manner as urine and produced a similar positive test. In another case a woman who had recently missed a menstrual period was being given a Friedman test to determine whether she was pregnant. We secured a sample of urine and ran a frog test simultaneously. The frog test was positive, the Friedman test was negative. Ten days later a second Friedman test gave a positive result. Parallel series are being run in several laboratories now.

During our very early studies we expected to accumulate a large number of positive tests in a short time by testing known late pregnancies. We ran 32 tests

in one day and found to our dismay that half of them gave negative results. This looked as if the test was not accurate and we were about to discontinue it when we recalled that studies on blood of pregnant mares and women had shown that the gonadotropic hormone increases very rapidly from conception to 30 days, decreases rapidly up to 90 days, and then continues at a low level around the threshold until delivery, at which time it seems to disappear. All of our tests made during the first trimester of pregnancy had given positive tests. This fact correlated with the abundance of the hormone in the blood during the first 90 days. During the third trimester the level of the hormone was so low that it might be above or below the threshold of sensitivity of the frog. We have tested a few cases during late pregnancy of women whose cycle was regular and who had accurate knowledge of dates and we found that the urine sample taken at the time when menstruation would have occurred gave very weak positive or even negative tests; whereas urine samples taken from the same women between menstrual dates gave strong positive results. This explained our 50 per cent false negatives in the last trimester. It seems that even though the woman is pregnant and bleeding does not occur, there is a lessening of the effect of the gonadotropic hormone during the period when menstruation would have occurred if she were not pregnant.

When testing an extremely early pregnancy, as ten days to two weeks, the number of sperm cells may be very few as compared with 30 days and a recheck should be made one week later which will usually give a strong reaction. It must be remembered, however, that the smaller number of cells is just as positive a test as a larger number. There is no such thing as being *slightly* pregnant.

Further clinical applications of this test are its use in abortions and missed abortions. Our first case was a natural miscarriage. A urine sample taken 6 hours after delivery of the foetus proved positive *indicating that the placenta was still adhering to the wall* of the uterus. Dilation of the cervix proved this to be true. The placenta was removed and a test of a urine sample taken 18 hours later gave a negative result. In the case of suspected missed abortions, where the placenta and uterine wall separate but delivery of the foetus does not follow, the frog test can indicate whether the separation has occurred or not. If the test is positive the placenta is still attached and alive. If the test is negative the placenta has separated, the child is dead and the undischarged mass can be removed. We hope to report more detailed information soon concerning clinical applications in therapeutic and pathological situations.¹

REFERENCES

- (1) Wiltberger, P. B. and D. F. Miller, "The Male Frog, *Rana pipiens*, as a New Test Animal for Early Pregnancy." *Science*, February 20, 1948, Vol. 107, No. 2773, p. 198.
- (2) MacLeod's "Physiology in Modern Medicine." C. V. Mosley Co., 1941, Ch. LXXVI; p. 1051.

¹Since this paper was prepared the following article has been published on some clinical applications of the Asheim-Zondek test:

ZONDEK, BERNHARD; FELIX SULMAN, AND RYVKA BLACK. "Hormonal Test for Foetal Death." *Jour. A. M. A.*, Vol. 136, No. 15, p. 965, Apr. 10, 1948.